Perspective

LPE-1, an orally active pyrimidine derivative, inhibits growth and mobility of human esophageal cancers by targeting LSD1

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A B S T R A C T

Histone lysine specific demethylase 1 (LSD1) plays an important role in epigenetic modifications, and aberrant expression of LSD1 predicts tumor progression and poor prognosis in human esophageal cancers. In this study, a series of LSD1 inhibitors were synthesized and proved to be highly potent against human esophageal squamous cell carcinoma (ESCC). Our data showed that these LSD1 inhibitors selectively suppressed the viability of esophageal cancer cell line (EC-109) bearing overexpressed LSD1. Among these, compound LPE-1 (LSD1 IC50 = 0.336 ± 0.002 μM) significantly suppressed proliferation, induced apoptosis, arrested cell cycle of EC109 cells at G2/M phase, and caused changes of the associated protein markers correspondingly. We also found that compound LPE-1 potently inhibited the migration and invasion of EC-109 cells. Docking studies showed that the cyano group formed hydrogen bonds with Val811 and Thr810. Additionally, the thioephene moiety formed arenë/H interaction with Trp761 residue. In vivo studies showed that compound LPE-1 inhibited tumor growth of xenograft models bearing EC-109 without obvious toxicity. Collectively, our findings indicate that LSD1 may be a potential therapeutic target in ESCC, and compound LPE-1 could serve as a lead compound for further development for anti-ESCC drug discovery.

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1. Introduction

Epigenetics is typically defined as the study of heritable changes in gene expression that are not due to changes in DNA sequence. In the past two decades, the epigenetic post-transcriptional modification mostly focused on the DNA, RNA, and histone modifications [1]. Among them, histone modifications, including methylation, acetylation, phosphorylation, ubiquination, sumoylation and so on, associate with the gene transcription or not [2]. Historically, histone methylation was believed to be an irreversible process until the histone lysine specific demethylase 1 (LSD1, also known as KDM1A) was first identified by Shi Yang’s group in 2004 [3]. LSD1 is an flavin adenine dinucleotide (FAD) dependent oxidative enzyme and specifically demethylates the mono- and di-methylated K4 and K9 of histone 3 [3,4], as well as some non-histone proteins such as

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E2F transcription factor (E2F1) [5], p53 [6], DNA methyltransferases (DNMTs) [4,7].

LSD1 has been proved to be tightly associated with tumorigenesis. Lim [8] et al. found that LSD1 was obviously overexpressed in human estrogen-receptor-negative breast cancer tissues and LSD1 siRNA-induced LSD1 knockdown could increase the expression of some proliferation-related genes, including p21, human epidermal growth factor receptor 2 (ERBB2), and cyclin-A2 (CCNA2). Lv et al. [9] reported that LSD1 was overexpressed in lung cancer tissues than normal lung tissues, and the level of LSD1 expression was negatively associated with the total survival time of non-small cell lung cancer (NSCLC) patients. Yu [10] and Chen [11] demonstrated that high expression of LSD1 was correlated with poor prognosis of patients with ESCC. Isamu Hoshino [12] further indicated that LSD1 inhibitors can inhibit cell growth by regulating some gene expression, such as Pleckstrin homology-like domain family B member 2 (PHLDB2), in ESCC. Our group reported two novel series of small-molecule LSD1 inhibitors that markedly suppressed invasion and migration of LSD1 overexpressed gastric cancer cells through regulating the process of epithelial-mesenchymal transition (EMT) [13,14]. LSD1 overexpression has also been observed in prostatic cancer [15], colon cancer [16], bladder cancer [17] et al. These data suggest that LSD1 may be a promising therapeutic drug target for cancer therapy.

Following our previous success in identifying new LSD1 inhibitors for anticancer treatment [13,14,18–23], we herein report the synthesis and antitumor activity of a series of LSD1 inhibitors against ESCC as well as the potential mechanisms. The most potent LSD1 inhibitor, compound LPE-1, selectively inhibited LSD1, suppressed proliferation, induced apoptosis, arrested the cell cycle at G2/M phase of EC109, and caused changes of the associated protein markers. Compound LPE-1 also suppressed the migration and invasion of EC-109 through inhibiting EMT process. In vivo studies showed that compound LPE-1 inhibited the growth of EC-109 xenograft tumors without obvious toxicity. Our findings indicate that LSD1 may be a potential therapeutic target in ESCC, and the compound LPE-1 could be a lead compound for further anti-ESCC drug discovery.

2. Materials and methods

2.1. Chemical

2.1.1. General

Reagents and solvents were purchased from commercial sources and used without further purification. Melting points were determined on an X-5 micromelting apparatus, and 1H NMR and 13C NMR spectrum were recorded on a Bruker 400 and 100 MHz spectrometer, respectively. High-resolution mass spectra were recorded on a Waters Micromass Q-T of Micromass spectrometer. The purity of all biologically evaluated compounds was determined to be >95% by reverse phase high performance liquid chromatography (HPLC) analysis.

2.1.2. Synthesis and characterization

2.1.2.1. General procedure for the synthesis of compounds 4a-l. The target compounds were synthesized as previously described [14]. As shown in Scheme 1, the 6-thiazole-5-cyano-2-thiouracil 4a-l were prepared from aromatic aldehydes 1 (1 mmol), ethylcynoacetate 2 (1 mmol), and thiourea 3 (1 mmol) in the presence of potassium carbonate in ethanol.

2.1.2.2. General procedure for the synthesis of compounds 5a-l. Without further purification, a mixture of the appropriate 2-mercapto- 2-dihydroxyrimidine derivatives 4a-l (1 mmol), the propargyl bromide (1 mmol), and anhydrous potassium carbonate (1 mmol) was refluxed in dry dioxane. Upon completion, as judged by TLC, phosphorus oxychloride was added dropwise with stirring while maintaining the temperature of the reaction mixture. Stirring was continued for additional 1 h. The cooled reaction mixture was poured on crushed ice and the separated solid was filtered off, washed with water, dried and crystallized from aqueous ethanol to yield the pure product.

2.1.2.3. General procedure for the synthesis of compounds 6a-l. To a well stirred solution of the thiosemicarbazide (1 mmol) in absolute ethanol (5 mL), equimolar amount of a solution of compounds 5a-l (1 mmol) in absolute ethanol (2 mL) was added. The reaction mixture was stirred and refluxed for 5 h. Upon completion, the precipitated product was filtered off and washed with ethanol to afford the crude product. The crude product was recrystallized from ethanol to yield the pure product.

Compound 6l is also named as LPE-1.

2.1.2.4. 4-chloro-2-(prop-2-yn-1-ythio)-6-[thiophen-2-yl]pyrimidine-5-carbonitrile (compound 6l). Yield 52%. Yellow solid. Mp: 119–120 ºC. 1H NMR (400 MHz, DMSO-d6, δ, ppm) δ 8.52 (dd, J = 4.0, 0.9 Hz, 1H, Ar-H), 7.78 (dd, J = 5.0, 0.9 Hz, 1H, Ar-H), 7.27 (dd, J = 4.9, 4.1 Hz, 1H, Ar-H), 3.99 (d, J = 2.6 Hz, 2H, −CH2−), 2.26 (t, J = 2.6 Hz, 1H, =C−H). 13C NMR (100 MHz, DMSO-d6, δ, ppm): δ 8.45, 8.42, 8.30, 8.27, 4.01, 4.00, 2.30, 2.29. HR-MS (ESI): Calcd. C13H8ClN3S2, [M + H]+ m/z: 291.9770; found: 291.9771.

2.2. Biological studies

2.2.1. Cell culture

Human esophageal cancer cell lines and human esophageal normal line were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Hyclone, USA) with 10% fetal bovine serum (FBS) (Hyclone, USA) and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO2 and 95% air at 37 ºC. All cell lines were purchased from the China Center for Type Culture Collection (CCTCC, China). For future pharmacological investigations, the compounds were prepared to the stock concentration of 10 mg/mL with dimethyl sulfoxide (DMSO), and the highest DMSO concentration in the medium (0.1%) did not have any substantial effect on the determined cellular functions.

2.2.2. Cell viability assay

Exponentially growing determined cells were seeded into 96-wells plate (Nest Biotechnology, China) at 4 × 103 cells per well. After 24 h incubation, remove the culture medium and replace fresh medium containing the candidate compound at gradient concentrations. The cells were incubated for another 72 h. Then, 20 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg/ml) was added to all wells and the cells were incubated for 4 h at 37 ºC. Discard the medium containing MTT, replace 150 μL DMSO, and agitate the plate until the dark violet (formazan) completely dissolved. The absorbance was measured.
using a microplate reader at a wavelength of 570 nm. Each concentration was analyzed in triplicate and the experiment was repeated three times. The average 50% inhibitory concentration (IC$_{50}$) was determined from the concentration response curves according to the inhibition ratio for each concentration.

### 2.2.3. Colony formation assay

Only about 1000 exponentially growing determined cells per well were seeded into 6-wells plate (Nest Biotechnology, China). After 24 h, the cells were incubated with different concentrations of compound LPE-1, for another 14 days. Then, the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min. Crystal violet staining was used to stain the cell for 30 min at 77°C. After that, wash away the staining with PBS until the colonies were clear enough.

### 2.2.4. Flow cytometric analysis of apoptotic analysis

Cells were seeded into 6-wells plate (Nest Biotechnology, China) at $2 \times 10^5$ cells per well. After 24 h incubation, the cultured medium was replaced with fresh medium containing the LPE-1 at different concentrations for another 48 h. Then, the cells were harvested and the Annexin-V-FITC/PI apoptosis kit (KeyGEN BioTECH, USA) was used according to the manufacturer’s instructions to detect apoptosis cells. Ten thousand events were collected for each sample and analyzed by (BD Bioscience, USA).

### 2.2.5. Hoechst 33258 staining

$2 \times 10^5$ per well exponentially growing determined cells were seeded in a 6-wells plate (Nest Biotechnology, China). After 24 h incubation, the cultured medium was replaced with the fresh medium with different concentrations of compound LPE-1 for another 48 h. Then, remove the medium, wash the cells with cold PBS gently, and fix the cells with 4% paraformaldehyde for 30 min. Cells were then stained with 5 μg/mL Hoechst-33258 (Beyotime Biotechnology, China) in PBS containing 0.5% Triton X-100 in the dark for at least 30 min. After the dye liquor and wash the cells adequately, apoptosis cells were examined and identified according to the condensation and fragmentation of nuclei by fluorescence microscopy under UV excitation.

### 2.2.6. Flow cytometric analysis of cell cycle distribution

After incubation with different concentrations of compound LPE-1 for 48 h, cells were harvested and washed with cold PBS for two times. Then, the cells were fixed with 70% ice-cold ethanol at 4°C overnight. The fixed cells were washed twice with cold PBS and stained with 1 mL PBS solution containing 1% Triton X-100, RNase and PI (KeyGEN BioTECH, USA) for 30 min under dark condition as previous. Ten thousand events were collected for each sample and analyzed by (BD Bioscience, USA).

### 2.2.7. siRNA transfection

About $1 \times 10^7$ EC-109 cells were seeded in a 6-well plate (Nest Biotechnology, China) and then incubated for another 48 h until the cells were 60–80% confluent at the time of transfection. Then prepare RNA-lipid complexes and add them to cells according to the manufacturer’s instructions (GenePharma, China).

#### 2.2.8. Migration and invasion assay

Wound healing assay, transwell assay and matrigel-coated transwell assay were conducted to evaluate the cell migration and invasion ability. All these experiments were carried out as previously published [13,14].

For the wound healing assay, cells were seeded in a 24-wells plate (CORNING, USA) at $1 \times 10^4$ per well. After 24 h incubation, the cell surface was scratched using a 10 μL pipet tip. Then, the cells were cultured with fresh medium containing 1% FBS and different concentrations of compound LPE-1 for 48 h, and photographed on an inverted microscope.

For migration assay, cells were seeded in Transwell 24-wells plate (CORNING, USA). 1% heat-inactivated FBS and 20% FBS were added into upper and lower chamber separately. Different concentrations of compound LPE-1 were added in the chamber. After 48 h incubation, remove the medium, wash the chambers with PBS and the migrating cells were fixed with methanol for 15 min and stained with Hoechst-33258. Each chamber was photographed using Thermo Fisher Cellomics High Content System.

For the matrigel-coated transwell assay, cells were seeded in Transwell 24-wells plate with Matrigel (BD Bioscience, USA). The medium, attractant, staining and cell counting method were the same as those of the Transwell assay.

#### 2.2.9. Western blotting analysis

Cells were cultured with different concentrations of compound LPE-1 for 48 h, and then the floating cells were collected and washed with cold PBS. Western blot was performed with the total lysates by cell lysis buffer [1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 25 mM Tris-HCl, 1% deoxycholic acid sodium salt, 1% PMSF] or histone purified with a kit (Epigentek, USA). Equal amounts of cell lysates were denatured, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to 0.22 μm nitrocellulose membrane. The membrane was blocked with PBS containing 5% nonfat milk for 2 h at room temperature and then incubated with primary antibody overnight at 4°C, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. The immunoblots were visualized by enhanced chemiluminescence kit (Thermo Fisher, USA).

Antibodies used were against histone H3K4me (ab194678, abcam, USA), H3K4me2 (ab194678, abcam, USA), H3K9me2 (ab194680, abcam, USA), H3K4me3 (ab71998, abcam, USA), total H3 (E1A6359, EnoGene Biotechnology, China), total H4 (E1A6355, EnoGene Biotechnology, China), pro-caspase3 (#9665, Cell Signaling, USA), cleaved-caspase3 (#9664, Cell Signaling, USA), pro-caspase7 (#12827, Cell Signaling, USA), cleaved-caspase7 (#8438, Cell Signaling, USA), Bcl-2 (E1A6139, EnoGene Biotechnology, China), Bax (E1A0120, EnoGene Biotechnology, China), LSD1 (ab37165, abcam, USA), Snai1 (#3879, Cell Signaling, USA), Slug (#9585, Cell Signaling, USA), N-cadherin (#13116, Cell Signaling, USA), Vimentin (#5741, Cell Signaling, USA), E-cadherin (#3195, Cell Signaling, USA).
2.2.10. Xenograft study

Animals were treated according to the protocols established by the ethics committee of Zhengzhou University and the in vivo experiments were carried out in accordance with the approved guidelines and approved by the ethics committee of Zhengzhou University. Female BALB/c nude mice (weighing 18–20 g and aged 5–6 weeks at the beginning of the assay) were purchased from Hunan Slack Scene of Laboratory Animal Co., Ltd. (Hunan, China). Xenograft model using human esophageal cancer cell lines, EC109, were established in BALB/c mice. Until the volume of tumors reached 100 mm³, mice were separated into control group and treatment group. The treatment group received compound LPE-1 at 50 mg/kg, p.o. per day for a period of 21 days during which the body weight was measured and tumor volume was determined by vernier caliper measurement at 3-day intervals. After the last day, the mice were euthanized and the tumors were isolated and weighted.
The expression level of LSD1 and histone methylation in EC-109 cells after treatment with compound LPE-1 were determined by Western Blot, and the total levels of histone 3 (H3) and histone 4 (H4) were used as loading control. * P<0.05 and ** P<0.01 were considered statistically significant compared with the control. Dates are mean ± SD. All experiments were carried out at least three times.

2.2.11. Molecular docking study

The protein complex was obtained from protein data bank (PDB code: 5L3E), and all water and ligand molecules were deleted. Hydrogen and partial charges were added by the protonate 3D program of MOE2014; Energy minimization of the ligands was carried out using energy minimize program of MOE. Default parameter settings generated by the program of MOE were used for docking.

2.2.12. Inhibitory evaluation of the compounds against LSD1

Inhibitory effects of the candidate compounds against LSD1 were evaluated as previously published [13]. Briefly, cDNA encoding LSD1(157–852AA) was cloned into pET28b to construct the plasmid pET28b-LSD1, and then the plasmid was transfected into BL21(DE). The recombinant was induced with 0.25 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 20 °C and purified with Ni-NTA resin. The compounds were incubated with the recombinant and H3K4me2, and the fluorescence was measured at an excitation wavelength of 530 nm and an emission wavelength of 590 nm with the addition of Amplex Red and horseradish peroxidase in order to evaluate the inhibition rate of the candidate compounds.
3. Results

3.1. Antiproliferative activity

Compounds LPE-1 and 6a-k (compounds from our in-house library [14]) were evaluated for their cytotoxic activity against four human cell lines: human normal esophageal cell line (Het-1A) and human esophageal cancer cell lines (EC-109, TE-1, EC-9706). Before testing the cytotoxic effects of these compounds, we first examined the expression levels of LSD1 in four different cell lines. As shown in Fig. 1, EC-109 cells possessed the highest LSD1 expression levels, followed by TE-1, EC-9706, Het-1A. We next evaluated the LSD1 inhibitory effect and antiproliferative activity of compounds 6a-k and LPE-1 against EC-109, TE-1 and EC-9706 cells and normal esophageal cell line Het-1A using 2-PCPA as the control drugs. As represented in Table 1, all compounds inhibited LSD1 at low micromolar levels, more potent than 2-PCPA (IC50 = 27.83 μM). Compound LPE-1 had improved LSD1 inhibitory activity (IC50 = 0.336 μM) compared to compounds 6a-k, suggesting the replacement of phenyl ring with heterocyclic ring system may be beneficial for the activity. Interestingly, the antiproliferative activity of these compounds against the tested cancer cells correlated with the LSD1 inhibitory effect. Compound LPE-1 (IC50 = 0.336 μM) displayed the best inhibitory activity against EC-109 cells, which had the highest LSD1 expression levels. In contrast, compound LPE-1 showed relatively weak inhibition toward TE-1, EC-9706 and Het-1A cells bearing relatively low LSD1 expression levels.

As shown in Fig. 2A, compound LPE-1 inhibited the proliferation of EC-109 in concentration- and time-dependent manner, while Het-1A was less sensitive against LPE-1. The difference in the antiproliferative activity promoted us to investigate the effect of LPE-1 on the colony formation. The results EC-109 showed that compound LPE-1 significantly inhibited proliferation of the single EC-109 cell, but was less sensitive toward Het-1A (Fig. 2B). Further flow cytometric analysis showed that compound LPE-1 induced G2/M phase arrest of EC-109 cell line in a concentration-dependent manner performed by (Fig. 2C).

3.2. Inhibition of compound LPE-1 on LSD1 activity

The favorable potency of compound LPE-1 against LSD1 (IC50 = 0.336 μM) encouraged us to investigate the effect of compound LPE-1 on LSD1 substrates in EC-109 (Fig. 3). After treatment for 48 h, the methylation levels of LSD1 substrates, H3K4me1/me2 and H3K9me2, increased dose-dependently, while H3K4me3, which is not the substrate of LSD1, was almost unchanged. What's more, the expression of LSD1 was also not affected. Those results suggest that the compound LPE-1 could inhibit the LSD1 activity in vitro, but not affect its expression.

3.3. Molecular docking studies

The favorable potency of compound LPE-1 toward LSD1 prompted us to perform the in silicon docking studies to rationalize the potency using the MOE2014 software (PDB code: 5L3E) [24]. The 2D interaction diagram (Fig. 4A) indicated that the cyano group formed hydrogen bonds with Val811 and Thr810. Additionally, the thiophene moiety formed arene-H interaction with Trp761 residue. The 3D binding models of compound LPE-1 in the active sited of LSD1 was represented in Fig. 4B. The docking results may provide a basis for further optimization.

3.4. Effect of compound LPE-1 on apoptosis and involved mechanism

To explore the apoptotic effect of compound LPE-1 on EC-109 and TE-1, we firstly examined the cell morphology changes after 48 h incubation with compound LPE-1 at indicated concentrations using Hoechst 33258 staining. The typical apoptotic markers, including cell rounding, chromatin shrinkage and apoptotic bodies were observed, especially at high concentrations (Fig. 5A). Next, we also performed apoptotic analysis with Annexin V-FITC/PI double staining and quantitated by flow cytometry. As shown in Fig. 5B, the percentage of apoptotic EC-109 cell (including early phase and late phase apoptosis) were increased up to 22.3%, 64.9% and 68.6% at the indicated concentrations, respectively, compared to the con-
trol (0.4%). While the Het-1A cell didn't show obvious apoptosis at the same concentrations, and the TE-1 cells, expressing LSD1 less than EC-109, needed higher concentrations of LPE-1 to obtain the significant apoptosis rate. Taken together, these date support the conclusion that the LPE-1 induced the apoptosis of EC-109 and TE-1 by targeting LSD1.

To further explore the mechanism of compound LPE-1 inducing apoptosis, the expression of key proteins involved the mitochondria-related apoptosis pathway was examined by Western Blot (Fig. 5C). The pro-apoptosis protein, Bax, was up-regulated in a concentration-dependent manner, while the anti-apoptosis protein Bcl-2 was down-regulated. EC-109 cells with down-

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**Fig. 5.** Compound LPE-1 induced apoptosis in EC-109 and TE-1 cells. (A) Apoptosis analysis with Hoechst-33258 staining after 48h of compound LPE-1 in EC-109 and TE-1 cells; (B) Quantitative analysis of apoptotic cells using Annexin V-FITC/PI double staining and flow-cytometry calculation; (C) Expressions of pro- and cleaved-caspase3, pro- and cleaved-caspase7, Bcl-2 and Bax were determined after 48h treatment with compound LPE-1; (D) Expression levels of LSD1, Bcl-2, and Bax were determined after LSD1 knockdown with siRNA for 48h * P<0.05 and **P<0.01 were considered statistically significant compared with the control. Dates are mean ± SD. All experiments were carried out at least three times.
regulated LSD1 expression levels caused by siRNA also showed upregulated Bax and down-regulated Bcl-2. Meanwhile, caspase-3/-7 activation were also observed after treatment with compound LPE-1. These findings indicted that LSD1 may be involved in the mitochondria-related apoptosis.

3.5. Effect of compound LPE-1 on cell migration and invasion

As reported previously, LSD1 was involved in epithelial-mesenchymal transition (EMT), which contributed for the migration and invasion abilities of cancer cells [25,26]. So we evaluated the migration ability of EC-109 and TE-1 by wound healing and transwell assay, the invasion ability was evaluated by matrigel-coated transwell. As shown in Fig. 6A, after scratched the EC-109 and TE-1 cells face, compound LPE-1 inhibited the would healing obviously, the transwell assay (Fig. 6B) also demonstrated the compound LPE-1 hindered the EC-109 and TE-1 cells migration through the biological membrane. The matrigel offered a simulant biological stroma, and EC-109 and TE-1 were blocked to invade through it in a concentration-dependent manner after treatment with compound LPE-1.

We also examined the expression of the typical proteins of EMT process. Fig. 6C showed that compound LPE-1 could up-regulate the epithelial cells’ biomarkers, E-Cadherin and Occludin, while the mesenchymal cells’ biomarkers, N-Cadherin and Vimentin, were down-regulated correspondingly. Then the upstream transcription factors, Snail and Slug, were also down-regulated after treatment with compound LPE-1, indicating that the compound LPE-1 might regulate the biomarkers of EMT process through inhibiting the expression of the transcription factors and cause the interference of EMT process ultimately. EC-109 cells with down-regulated LSD1 expression levels by siRNA also showed upregulated the epithelial cells’ biomarkers, E-Cadherin and Occludin, while down-regulated the mesenchymal cells’ biomarkers, N-Cadherin and Vimentin (Fig. 6D).
Fig. 6. Compound LPE-1 blocked EMT process in EC-109 and TE-1 cells. (A) Wound healing assay. (B) The migration and invasion ability of EC-109 and TE-1 cells after treatment of compound LPE-1 was reflected using transwell and matrigel-coated transwell; (C) Expressions of Snail-1, Slug, N-Cadherin, Vimentin, E-Cadherin, Occludin were determined after 48 h treatment of compound LPE-1. (D) Expression levels of LSD1, N-Cadherin, Vimentin, E-Cadherin, and Occludin were determined after LSD1 knockdown with siRNA for 48 h. *P < 0.05 and **P < 0.01 were considered statistically significant compared with the control. Dates are mean ± SD. All experiments were carried out at least three times.
3.6. In vivo antitumor study

Since the obvious inhibitory activity of compound LPE-1 against EC-109 cell, we also evaluated the in vivo antitumor effect of compound LPE-1 on xenograft model bearing EC-109 cells by subcutaneous implantation. After the treatment of compound LPE-1, the body weight of mice, the tumor weight and the tumor volume were measured and recorded every three days. As shown in Fig. 7A, B, and D, compound LPE-1 inhibited tumor growth remarkably, while the body weight was almost unchanged (Fig. 7C), suggesting the antitumor efficacy and low global toxicity.

4. Discussion

Esophageal cancer, the eighth most common cancer worldwide, has poor prognosis, the 5-year survival rate for esophageal squamous cell carcinoma (ESCC) is less than 20% [27,28]. Therefore, novel prognostic and molecular targets for therapeutic intervention are urgently needed. Many researches have reported that LSD1 is highly expressed in several types of cancer, such as estrogen-receptor-negative breast cancer [8,29,30], prostate cancer [26,31], bladder cancer [17], colorectal cancer [16], gastric cancer [13,14], lung cancer [9] and others. Meanwhile, recent work suggested that inactivation of LSD1 by small-molecule inhibitors or siRNA may have anticancer therapeutic potential [12–14,23,26,29,32–34]. Therefore, researchers have devoted to testifying the role of LSD1 in ESCC [10–12,35].

From our in-house small-molecule library, compound LPE-1 was identified as a new LSD1 inhibitor, which inhibited LSD1 at submicromolar levels (IC50 = 0.336 μM). Compound LPE-1 selectively suppressed growth of LSD1 overexpressed EC-109 cells, while for EC-9706, TE-1, and Het-1A cells with low LSD1 expression levels, less cytotoxic effect was observed. Compound LPE-1 induced apoptosis, arrested the cell cycle at G2/M phase against EC109, and...
exhibited promising antitumor efficacy toward EC-109 xenograft model. Additionally, after treatment of EC-109 cells with compound **LPE-1**, the methylation levels of LSD1 substrates (H3K4me1, H3K4me2 and H3K9me2) were up-regulated in a concentration-dependent manner, while the methylation level of H3K4me3 and expression of total LSD1 were unchanged compared to H3 and H4. There data suggested that the compound **LPE-1** specifically inhibited LSD1 activity at the cellular level and did not change the expression of LSD1.

The increased motility and invasiveness of tumor cells are reminiscent of the processes at the epithelial-mesenchymal transition (EMT), which contributes to the normal morphogenetic events such as embryonic development, tissue remodeling, wound healing and metastasis, but also the malignance of cancer cells. The EMT process in cell differentiation and behavior is mediated by key transcription factors, including Snail, zin-finger E-box-binding (ZEB) and basic helix-loop-helix transcription factors. Their expression is activated early in EMT process, and they further define the EMT transcription program and drive EMT process [36–39]. Recently, it has been reported [25] that the transcription factor Snail repressed E-cadherin expression by recruiting LSD1 to its promoter, which contributed to cancer cell invasion. Additionally, Bruno Calabretta’s group [40] found that blocking Snail-LSD1 interaction by Parnate, an enzymatic inhibitor of LSD1, could suppress the invasiveness of cancer cells.

Therefore, we hypothesized that the potent LSD1 inhibitor, compound **LPE-1**, might suppress the migration and invasion of EC-109 through blocking the EMT process. The data of some intuitionistic assays, such as wound healing assay, transwell and matrigel-coated transwell assay, demonstrated that the compound **LPE-1** could obviously repress the activity of migration and invasion against EC-109. Further investigation showed that compound **LPE-1** could up-regulate the epithelial cells' biomarkers, E-Cadherin and Occludin, while the mesenchymal cells' biomarkers, N-Cadherin and Vimentin, were down-regulated.

Interestingly, the upstream transcription factors, Snail and Slug, were also down-regulated after treatment with compound **LPE-1**. It has been recognized that LSD1 is essential for Snail1-mediated transcriptional repression and for maintenance of the silenced state of Snail target genes in invasive cancer cells. The N-terminal SNAG domain of Snail1 and the amine oxidase domain of LSD1 were required for their interaction [37]. Moreover, blocking the interactions between LSD1 and Snail/Slug inhibits cancer cell invasion [40]. Therefore, the different observations may be related to unknown mechanisms, which require further investigations. Collectively, these results suggest that compound **LPE-1** could reverse the EMT process, which might contribute to its antitumor effect in vitro and in vivo on EC-109 cell line and xenograft mice models.

In conclusion, our study identified a series of compounds based on pyrimidine-thiourea hybrid and its analog **LPE-1** as new potent LSD1 inhibitor. Especially, compound **LPE-1** displayed obvious anti-cancer activity in vitro and in vivo against LSD1 overexpressed EC-109. Further investigations demonstrate that compound **LPE-1** regulated the activity of LSD1 and then reversed the EMT process, which finally induced the inhibition of migration and invasion against EC-109. Collectively, we identify that LSD1 may be a potential therapeutic target in ESCC, and the compound **LPE-1** could be a lead compound for further anti-ESCC drug discovery.

**Conflict of interest**

The authors declare no conflicts of interest.
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2017.05.025.

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